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IN-VIVO EVALUATIONS OF THE STEM BARK OF *COMBRETUM MOLLE* "*R.BR/G. DON*" (KEAY, 1989) FOR ANTHELMINTIC PROPERTIES.

[1]Simon M.K., [2]Ajanusi J.O., [2]George B.D., [3]Abubakar M.S., [1]Meduna J.A
[1]Federal College of Wildlife Management, New-Bussa, Niger State
[2]Department of Veterinary Parasitology and Entomology, Faculty of Veterinary Medicine, A.B.U, Zaria
[3]Department of Pharmacognocy, Faculty of Pharmaceutical Sciences, A.B.U, Zaria

ABSTRACT
The anthelmintic activity of partitioned portions of the crude methanolic extract of *Combretum molle* was evaluated *in-vivo* in rats' model experimentally infected with *Nippostrongylus*. The crude methanolic extract of the plant was obtained after extraction with absolute methanol; and further partitioned between three solvents (i.e. petroleum ether, chloroform and N-butanol). The extract portions (with the exception of petroleum ether) were tested for anthelmintic activity against *Nippostrongylus* in rats. Phytochemical screening conducted on the extracts revealed constituents that have anthelmintic effect such as; alkaloids, steroids, saponins, tannins, flavonoids and glycosides. The anthelmintic activity was assessed by comparing the number of worms recovered from rats treated with the portions to those from non-treated infected controls. This study considered deparasitization rate of 70 % or greater as significant. The aqueous methanol, chloroform and N-butanol portions produced significant ($p<0.05$) deparasitization rate of 86.98 %, 79.20 % and 72.72 % respectively when a maximum tolerated dose of 1000 mg/kg^{-1} was administered.
Thus the extracts are recommended for further studies in ruminant to validate their efficacy in them.

KEYWORDS: *in-vivo,* anthelmintic, *Combretum molle, Nippostrongylus braziliensis*

INTRODUCTION

Parasitic nematodes are among the most common and economically important infectious disease organisms of grazing livestock especially ruminants around the world (Perry *et al.*, 2002; Alawa *et al*., 2001).

In Nigeria, helminthosis can result in mortality rates exceeding 50 % with an estimated loss of $144 million annually in animal products and services (Jegede *et al*., 2006; Perry and Randolph, 1999).

Conventional anthelmintics are good remedies for the control of livestock parasitosis, but constraints such as high cost, adulteration, resistant strain development, residual effect on man and the declining funding of veterinary services especially in developing countries are limiting the use of such drugs (Ademola *et al.*, 2004; Uza *et al.*, 1996).

However, efforts are now directed towards screening herbal medicinal plants claimed by traditionalists and pastoralists as remedies for helminthosis in both *in vitro* and *in vivo* studies (Githiori *et al., 2003b,* Alawa *et al.*, 2003; Ademola *et al.*, 2004).

Plants produce valuable organic substances that have potential value in the treatment of diseases. It is necessary therefore to investigate such plants and to isolate and identify their active principles (Abdul Ghani, 1990)

Moreover; the rational use of drugs requires the standardization of their crude forms, dosage formulation as well as the determination of their mode of action and any toxic manifestations which they may produce (Vercruysse *et al.*, 2001; Ibrahim *et al*., 1984).

Combretum molle '*R.Br/G.Don'* (Keay, 1989) is a tree distinguished by its rough bark and dense crown. It is commonly referred to as wuyan damo (Hausa), damoruhi (Fulani) and aragba (Yoruba); the common English name is obscured. There was no reported medicinal use of this plant apart from the purported claims of the nomads and the pastoralists that its stem bark possessed anthelmintic activity in animals when given as concoction (Alawa *et al*., 2000; Atawodi *et al.*, 2000).

In view of public concern over perceivable drug residues in animal products, the increasing prevalence of anthelmintic-resistant strains of nematodes, the rising cost of such organic substances, there is an urgent need for development of sustainable alternatives to conventional anthelmintics in ruminant production system. This can be achieved through research into newer anthelmintics from natural resources. Thus this work is aimed at assessing the *in vivo* anthelmintic effect of the crude methanolic extracts (CME) and the partitioned portions of the bark of *Combretum molle* against adult *Nippostrongylus braziliensis* in experimentally infected rats with the view of extrapolating the findings in ruminant production system.

Also to identify the most active portion of the partitioned extracts, with the view of providing scientific basis for their use in ethno-veterinary practices.

MATERIALS AND METHOD

The study was conducted in April, 2007 at the Department of Parasitology and Entomology, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria, Nigeria.

Plant collection, identification and preparation

Five kilogram's of the stem barks of C*ombretum molle* were collected from Zaria, Nigeria and properly identified by a botanist, and a voucher number (V/No 297) was given. Samples were sun dried, powdered using mortar and pestle into fine powder and sieved as described by Onyeyili *et al* (2001).

Five hundred grams of the powdered sample was extracted exhaustively with 2.5litres of absolute methanol in a soxhlet apparatus (Youn *et al*, 2003; Onyeyili et al, 2001).The extract was concentrated to dryness in a vacuum using a rotary evaporator coupled to a thermo-regulator (Hordegen *et al*, 2003).

Twenty grams of the dried crude methanolic extract was further partitioned in a step-wise separation process in petroleum ether, chloroform, and N-butanol using a separating funnel (Assis *et al*, 2003).

The solvents were evaporated leaving the dried portions which were then tested *in-vivo* for anthelmintic activity against *N. braziliensis* in a rat model (Ibrahim *et al*., 1984; Suleiman *et al*, 2005)

Phytochemistry

Phytochemical screening of the crude methanolic extract and the partitioned portions was conducted using standard techniques described by Trease and Evans (1983) and Brain and Turner (1975).

Experimental animals (Rats)

Sixty albino Wister rats (6 to 7 weeks old) of both sexes and weighing between 100 to 160 g were used in the study.

The rats were acclimatized to laboratory conditions for two weeks and fed on commercially prepared feed; water was given *ad lib*.

Forty two rats used for the anthelmintic study were dewormed using albendazole at 200 $mgkg^{-1}$ two weeks before experimental infection (Suleiman *et al.*, 2005).

Evaluation of maximum tolerated dose (MTD) of the crude methanol extracts
Due to lack of information on the precise dosage of the plants preparation used by traditional herdsmen and the pastoralists, a maximum tolerated dose (MTD) experiment described by Lorke (1983) was carried out to determine the experimental dose of the plant using the crude methanolic extract in eighteen rats.

Post-mortem findings
Rats were randomly selected one from each of the dose levels of the preliminary maximum tolerated dose trial, one from each of the partitioned portions after the experimental treatment and salvaged for gross and histological changes on the visceral organs resulting from the administration of the plants extracts (Suleiman *et al*., 2005).

Experimental infection/design
Forty two worm-free rats were infected by injecting subcutaneously in the cervical region with 200 viable third stage larvae (L3) of *N. braziliensis* in .2 ml of water using an 18-gauge needle attached to an insulin syringe (Suleiman *et al.*, 2005).

Five days post infection, rats were screened for evidence of infection through faecal screening using simple floatation technique (Soulsby, 1982). Rats not shedding ova of *N. braziliensis* were excluded from the experiment.

The infected rats were randomly allocated to three (3) treatment groups (A-C).
Group 'A' had six rats and treated with albendazole at 200 $mgkg^{-1}$ body weights as a positive control group (Suleiman *et al.*, 2005).

Group 'B' (having 24 rats), were sub-divided into four groups of six rats each and treated with the crude methanolic extract, chloroform, N-butanol and aqueous methanol dried portions at 1000 $mgkg^{-1}$ (Vercruysse *et al.*, 2001).

Group 'C' having 12 rats and were subdivided into two groups of six rats each and given water and propylene glycol at 5 $mlkg^{-1}$ as negative controls (Ibrahim *et al*., 1984).

Worm counts
On Day 2-post treatment, all rats were fasted for 24 hours, salvaged for adult worm count, using the WAAVP guides (Powers *et al.*, 1982).

The first 15 cm of the small intestine was removed, cut longitudinally and placed between two clean 20 cm glass slides. The section was examined at x40 magnification of a dissecting microscope. Visible worms were counted and recorded (Suleiman *et al.*, 2005).

The portion that showed the highest reduction in worm count and did not produce any behavioral changes in the rats was considered to be the most active portion (Githiori *et al.*, 2003).

Percentage efficacy
Percentage efficacy (deparasitization) of the crude methanol extract and the various portions of each plant was calculated according to the method used by Cavier (1973). The percentage efficacy was calculated using the formula:

$$\% \text{ Efficacy} = \frac{N-n}{N} \times 100\%$$

N= number of worms counted in the placebo-treated rats
n= number of worms counted in the plant extracts or albendazole-treated rats.

Statistical analysis
Means of data obtained were analyzed statistically using the software package for Graph-Pad prism (version 4.0/2003).
Statistical significance for the anthelmintic effect of the crude methanol extract, chloroform, N-butanol portion and aqueous portion was assessed by ANOVA.

Subsequently Borferroni's multiple comparison test was carried out to determine the most active portion.
P value < 0.05 was considered significant.

RESULTS
Methanolic Extraction of plant material and solvent partitioning of crude methanolic extracts
One hundred and fifty grams (30 %) was obtained after subjecting of 500 g powdered plant material to extraction with absolute methanol. Using petroleum ether, chloroform and N-butanol as separating solvents, the respective yields following partitioning of 20 g crude methanolic extract were 0 g (0 %), 5 g (25 %), 7 g (35 %) and 8 g (40 %) as the aqueous methanol portion

Phytochemical screening
The phytochemical screening revealed that the crude methanolic extract of the plant had alkaloids, steroids, saponins, carbohydrate, flavonoids, tannins and cardiac glycosides as constituents; the petroleum ether portion of the plant revealed the presence of only alkaloids and steroids; while the chloroform portion, when screened, showed the presence of alkaloids, steroids, flavonoids, tannins and cardiac glycosides. The screening of the N-butanol portion revealed the presence of carbohydrate, in addition to the constituent seen in the chloroform portion. The screening of the aqueous methanol portion revealed the presence of steroids, flavonoids, tannins, carbohydrate and cardiac glycosides.

Table 1: Phytochemical screening for the crude methanol extract and various portions of *A. africana.*

	Portions				
Constituents	crude methanol extract	pet ether	chloroform	N-butanol	aq methanol
Alkaloids	+	+	+	+	-
Cardiac glycoside	+	-	+	+	+
Carbohydrate	+	-	-	+	+
Flavonoids	+	-	+	+	+
Saponins	+	-	-	+	+
Steroids	+	+	+	+	+
Tannins	+	-	+	+	+

Key
Present (+)
Absent (-)

Determination of maximum tolerated dose.
The crude methanolic extract at a dose range of 10 to 1000 $mgkg^{-1}$ did not cause any visible toxic effect in the rats- the rats were active 6 to 12 h after recovering from the stress of the administration procedure. On the other hand, at a dose range of 1600 to 5000 $mgkg^{-1}$, there were indications of toxicity – weakness after 24 hrs of administration, a case of mortalities at 24 h in the group given 2900 $mgkg^{-1}$, and two mortality at 12 h in the group given 5000 $mgkg^{-1}$ (Table 2), thus 1000 $mgkg^{-1}$ was chosen as experimental dose.

Table 2: Maximum tolarated dose/toxicity of crude methanol extract of *C. molle*

• Dose(mg/kg⁻¹)	10	100	1000	1600	2900	5000
Initial number of rat	3	3	3	3	3	3
Mortality	0	0	0	0	1	2
Observation	a	a	a	b	c	d
Inference	-	-	-	+	++	+++

Key

a = rat active 6-24 hrs and beyond, b = rats showed weakness for more than 24 hrs, c = rats showed weakness for more than 48 hrs, d = rats showed weakness for more than 7 days, - = no sign of toxicity, + = slightly toxic,

++ = less toxic, +++ = toxic.

Post mortem findings

There were no gross pathological and histological lesions on the visceral organs resulting from the administration of the tested doses of the crude methanolic extract and various portions.

Anthelmintic effect of extracts on *N. braziliensis*

Rats that had oral infection of 200 L3 followed by treatment with crude methanolic extract at 1000 mg/kg⁻¹ had a mean worm count of 6.17; while those treated with chloroform, N-butanol and aqueous methanol portions at 1000 mg/kg⁻¹ each and albendazole at 200 mg/kg⁻¹ had mean worm count of 3.0, 3.5, 1.67 and 0 respectively; compared to the mean worm count of 12.83 and 12.0 from the negative controls treated rats. The extracts produced percentage deparasitization of 48.61 %, 75.0 %, and 72. 72% and 86.98 % for crude methanolic extract chloroform, N-butanol and aqueous methanol respectively.

Table 3: Worm count and percentage deparasitization 7 days after treatment with crude methanol extract and fractions of *C. molle*

•		Worm cout after treatment with					
• Rat	CME (1000mgkg⁻¹)	Chloroform (1000mgkg⁻¹)	N-butanol (1000 mgkg⁻¹)	Aqueous (1000 mgkg⁻¹)	Albendazole (200 mgkg⁻¹)	Placebo1 (5 ml-water)	Placebo2 (5 ml-p. glycol)
• 1	7	2	6	2	0	4	6
• 2	2	3	0	0	0	17	16
• 3	7	3	5	0	0	18	15
• 4	8	4	3	4	0	12	10
• 5	7	3	3	2	0	14	11
• 6	6	3	4	2	0	12	14
Mean ± SD	6.17 ± 2.11b	3.0 ± 0.63a*	3.5 ± 2.09a*	1.67 ± 3.37a*	0.0 ± 0.0a*	12.83± 5.0b	12.0 ± 3.74b
% DPZ	4 8.61	75.0	72.72	86.98	100	0	0

Mean with * within the column are significantly different at $p<0.001$, while those with the letter ª and ъ show no significant difference between their means at $p>0.05$ as determined by Borferroni's multiple comparison test. %DPZ= percentage deparasitization

Compared to the negative controls, the crude methanolic extract and the various portions albendazole gave 100 % deparasitization

The deparasitization produced by the chloroform, N-butanol and aqueous methanol portions were significant ($p<0.05$) when compared to that produced by the negative control rats, while the deparasitization produced by the crude methanolic extract was non-significant ($p>0.05$) (Table 3).

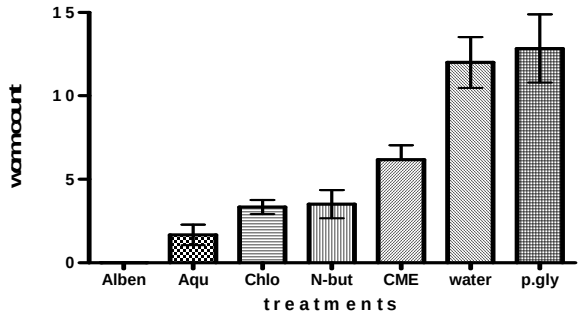

Fig 1: Mean ± SD of worm count after treatment with the various fractions of *C. molle* extracts, albendazole and the placebos

DISCUSSION

Recent harmonizations on anthelmintic efficacy guidelines in ruminants have indicated that for a drug to be considered efficacious, a 90 % reduction in total worm count should be achieved (Vercruysse *et al*., 2001). However, the *in-vivo* anthelmintic effect of the plant extracts is unknown. Thus, it was considered 'a priori' that the efficacy of the extracts would be biologically significant if a reduction in total worm count above 70 % occurred (Githiori *et al*., 2003).

Rats treated with chloroform, N-butanol, and aqueous methanol portions showed anthelmintic activity. The aqueous methanol portion showed the highest reduction in total worm count 86.98 %. This was followed by chloroform and N-butanol portions with reduction in total worm count of 75.0 % and 72.20 % respectively. Treatment with the crude methanolic extract did not produced the required significant biological reduction in worm count in comparison with the untreated control groups. The results of this study also demonstrated that the parasites *N. braziliensis* was highly sensitive to albendazole with complete deparasitzation at a dose of 200 mg/kg^{-1} (Suleiman *et al*., 2005).

The outcome of the phytochemical screening revealed that plant extracts have constituents including tannins, alkaloids, flavonoids, cardiac glycosides and steroids which may have anthelminthic activities (Athanasiadou *et al*., 2001, 2005; Gamenara *et al*., 2001; Lahlou., 2002; Onyeyili *et al*., 2001; Lateef *et al*., 2003; Prasharth *et al*., 2001) Kahiya *et al*. (2003) in *in-vitro* studies reported that condensed tannins from the leaves extract of *Acarcia nitotica* inhibited the development of *H. contortus* larvae from goats. Tannins polyphenols from bryophytes were shown to have anthelmintic activity against *N braziliensis* (Gamenara *et al*., 2001). Athanasiadou *et al*. (2001) in *in-vitro* and *in-vivo* studies reported the anthelmintic activity of condensed tannins extracted from *Quebracho* on the larvae of *H. contortus*, *Teladorsagia circumcinta* and *Trichostrongylus vitrinus.* Anthelmintic activity of tannins is attributed to their capacity to binds to free proteins available in the gastrointestinal tract of the host reducing nutrients available to the parasites resulting into starvation and death (Athanasiadou *et al*., 2001). Also tannins are capable of binding with the glycoproteins on the cuticle and leading to death of the parasite (Thompson and Geary, 1995). Also tannins have vasoconstriction effect and could be advantageous in preventing worm implantation onto the mucosa of the gastrointestinal tract thus making their expulsion from the GIT easier (Aguwa and Nwako, 1988; Min and Hart, 2003).

Tannins-containing plants increase the supply of digestible protein by animals thus improving the immunity against gastrointestinal parasites (Coop and Kyriazakis, 1999; Coop and Holmes, 1996). Tannins contain mixtures of phenols which combine with plasma proteins rendering them resistant to proteolytic enzymes secreted by the worms (Mitcell *et al*., 1983; Kahn and Diaz-Hernandez, 2000). It is therefore reasonable to assume that the herdsmen/pastoralists claims may be right since the plants used in this study contain tannins and could have had anthelmintic effect similar to the ones earlier described.

Hashizume *et al.* (1978) reported that flavonoids offer some protection against ulcer by increasing capillary resistance and through improved microcirculation which renders the cell less injurious to predisposing factors such as worms. Flavonoids are also believed to stimulate intestinal motility similar to that produced by acetylcholine (Akendenque, 1992), there by causing rapid worm expulsion from the gastrointestinal tract.

Lahlou (2002) reported that flavonoids are phytochemicals that have anthelmintic effect. Having identified flavonoids in almost all the portions used in this study, it is possible that they had a significant anthelmintic effect on the *N. braziliensis* resulting in the observed deparasitization.

In *in-vitro* and *in-vivo* studies, Al-Qarawi *et al.* (2001) reported that alkaloids extracted from both the latex and leaves of C*alotropis procera*, were effective in inhibiting the exsheathment of L3 of *H. contortus* to L4 in sheep. Lateef *et al*. (2003) also reported that alkaloids and their glycosides extracted from the roots of *Adhatoda vestica* were effective against mixed gastrointestinal infections in sheep. Also, Onyeyili *et al.* (2001) reported that tannins and alkaloids, the active principles of *Nauclea latifolia* bark were effective against mixed infections in sheep. The present study has shown that alkaloids are present in all the portions except the CME. It is possible that the presence of alkaloids had a role in the significant deparasitization observed. Conversely, the absence of alkaloids in the CME may have accounted for the very poor and insignificant deparasitizatioin observed with this portion.

Glycosides extracted from the root of *Adhatoda vestica* were found to be effective against mixed gastrointestinal nematode infection in sheep (Lateef *et al*., 2003). Also this compound has been shown to induce tonic contraction that resulted in the expulsion of the worms from rat's gastrointestinal tract (Kim *et al*., 1992). Cardiac glycosides were identified in the plant extracts used in this study and could have had the same effect.

Steroids were identified as active principle in *Butea monosferma* seed and reported to have *in-vitro* anthelmintic activity against adult *Caenorhabditis elegans*, a free-living nematode (Prasharth *et al*., 2001). However, it is unknown whether the steroids identified in all the portions of both plants could have had the same effect *in-vivo*.

The maximum tolerated dose trials or otherwise referred to as dose determination studies (Vercruysse *et al*., 2001) were carried out on the premise that the plant extracts under investigation had no alternative data to support any intended dosage.

In this work, the injurious dose and the maximum tolerated dose were determined. The plant extracts produced lethal effect at a dose of 1600 to 5000 $mgkg^{-1}$. However there was no sign of toxicity at a dose of 10-1000 $mgkg^{-1}$. Therefore 1000 $mgkg^{-1}$ was selected as the maximum tolerated dose as well as the experimental treatment dose.

CONCLUSION

Results from this study demonstrated that the aqueous methanol, chloroform and N-butanol portions of the plant extracts were effective against experimental *N. braziliensis* infection in rats at a non-toxic dose of 1000 $mgkg^{-1}$. The chemicals believed to constitute the active principles in the plant have significant anthelmintic efficacy whereas albendazole was found to be highly efficacious. The investigation of the effects of chemical compounds on helminths from natural products is fundamentally important for the

development of new anthelmintics drugs, especially in view of the vast worldwide flora. Thus a quality controlled extraction of *C. molle* and the isolation of their bioactive compounds could be a promising alternative to conventional anthelmintics for the treatment of gastrointestinal helminths of ruminants in the future.

Unknown factors may also have an influence on the anthelmintic activity of these plants. For instance, the rats may have generated a strong T. cell-depedent immune response that brings about expulsion of the worms from the intestine (Mitcell *et al*., 1983)

More detailed studies are needed to isolate, characterize and evaluate the active components and the mechanism of action of the identified active principles. Also preliminary studies on the toxicity, evaluation of the effect *in-vivo* against economically important gastrointestinal nematode species and the establishment of adequate doses for sheep, goats and cattle are needed.

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Where is Githiori 2003a? if none delete letter ‘b’ from 2003b and do same in the text

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Corresponding Author:
Simon M.K.
Federal College of Wildlife Management, New-Bussa, Niger State